Procedures for the Quantification of Human and Male Nuclear DNA

1 Scope

These procedures apply to DNA personnel who perform quantification to determine the quantity of amplifiable human and male nuclear DNA (nDNA) detected in a sample and DNA personnel that perform the associated quality control procedures. The DNA Casework Unit (DCU) and Scientific and Biometrics Analysis Unit (SBAU) use Sample Tracking and Control Software (STACS) and robotic workstations to automate the set-up of the quantification (aka quant) plates.

2 Equipment/Materials/Reagents

Equipment/Materials

- Tecan robot model 'Freedom EVO'
  - Tecan EvoWare software, version 2.3 or higher
- 7500 Real-Time PCR System, Applied Biosystems
  - HID Real-Time PCR Analysis Software vs 1.2 or higher
- STACS, version 5.0 or higher
- General laboratory supplies (e.g., pipettes, tubes, vortex, centrifuge)
- Microcentrifuge tubes (robot compatible) (e.g., 2mL screw cap for Master Mix and 1.5mL screw cap for standards/samples)
- Speed-Vac, Vacufuge Concentrators, or equivalent
- 96-well Plates, Applied Biosystems MicroAmp® optical or equivalent
- Clear plate seals
- Thermal Microplate Sealer

Reagents

- Quantifiler® TRIO DNA Quantification Kit
  - Prepared Quantifiler® TRIO DNA standard calibrator samples, 1:10 (~10 ng/µl) and 1:50 (~2 ng/µl)
- Quantifiler® Automation Enhancer
- 3% bleach (reagent grade or equivalent)
- 10% bleach (reagent grade or equivalent)
- Isopropyl alcohol, 70%
- Purified water or equivalent, available at laboratory sinks
- Water (reagent grade or equivalent)
- Roboscrub solution (Liquinox™ or equivalent)
- Standard Reference Material (SRM) 2372 Human DNA Quantitation Standard (or equivalent)

3 Standards and Controls

A Master Mix control and the prepared Quantifiler® TRIO DNA standard calibrator samples will be run on each plate. Evaluation of these control samples can be found in the Data Evaluation section of this procedure.

The reagent blank(s) (RB) associated with each extraction batch are quanted to determine the RB with the greatest (if any) signal.

4 Procedures

Refer to the DNA Procedures Introduction (i.e., DNA QA 600) and follow applicable general precautions and cleaning instruction. Ensure the appropriate fields (i.e., instruments, reagents) in STACS are completed, as necessary.

For water that will come into contact with the DNA samples (e.g., for dilutions), reagent grade, or equivalent, water will be used. The purified water, available via faucets (typically labeled DE) at the laboratory sinks, is used for Tecan operation and is also called Tecan system liquid.

4.1 Concentrating Extracted Samples Using the Speed-Vac or Vacufuge

4.1.1	Samples may be concentrated using a Speed-Vac or Vacufuge. • Samples from questioned items and corresponding RBs are generally reconstituted with reagent grade water, vortexed and quick spun prior to quantitation. Female fractions from vaginal swabs (and similar sample types) are generally not concentrated. • Known samples are generally not concentrated. The volume of water to use to reconstitute, typically 15 µL or 25uL, will be recorded in STACS. This volume is determined by the type of sample or as requested by the examiner. The volume used for the RB must be the same or less than the volume used for the associated samples.	

The Speed-Vac/Vacufuge flask should be emptied as needed, and the flask seal should be tight. The Speed-Vac/Vacufuge should be turned on ~45 minutes prior to use.

Ensure the gasket on the centrifuge is in its proper position and that the rotor is properly tightened prior to sample processing.

On the Speed-Vac with the heat set to "High", a 50 µL extract may take ~30-40 minutes to dry and a 100 µL extract may take ~60 minutes to dry. Samples should not be dried on "High" for more than four hours (maximum starting volume of ~400 µL).
On the Vacufuge with a setting of 60ºC, a 50 µL extract takes ~45 minutes.

4.2 Preparing the Tecan Robotic Workstation

4.2.1	Ensure the Tecan is prepared to run: Prior to daily use: • Make ~100mL of 3% bleach to replace in front trough. • Clean the outside of the Tecan tips with 70% isopropyl alcohol • Decontaminate the Tecan work deck with 10% bleach • Run the daily start up script Prior to each run: • Check system liquid (i.e., purified water) level and replace/refill the carboy if needed. *When a carboy is refilled, it should be allowed to de-gas overnight before use.* • Check volume of waste container and empty if needed As needed: • Clean barcode scanners with a lint-free cloth	

The daily start up script prompt "Check syringes and tips" refers to checking that the tubing and syringes (plunger lock screws) are tight and not introducing air bubbles, and that the tips are tight, free of clogs, and not leaking.

4.3 Preparing the Tecan Deck and Reconstituting samples

The below steps may be performed in any order prior to running the Tecan robot for Quant TRIO plate preparation.

Positions of materials may vary between instruments. The robotic script will direct the placement.

4.3.1	Bleach Rack: • Ensure the 3% bleach solution in the front trough was replaced prior to first daily use.	

4.3.2	**If using the Tecan to reconstitute samples:** Water Rack: • Ensure center trough has ~200 mL reagent grade water, replaced prior to each batch of samples for a quant plate.	

4.3.2.1	Based on the volume of water to reconstitute recorded in STACS, load the concentrated sample tubes in positions 1 through 16 in a sample rack. Use additional sample racks as needed for each group of tubes for a volume. Run the appropriate TECAN script to add reagent grade water to the sample tubes. Repeat as needed for each group of tubes based on volume.	

4.3.2.2	Cap, vortex, and quick spin the tubes prior to preparing the sample rack(s) for the Quant Trio Plate Preparation	

4.3.3	Plate Rack: • Place a 96-well plate into a base. • Place into the front position of the plate rack with the A12 notch at back right. • Ensure a quant batch barcode label is on the right side of the base or the plate, as appropriate.	

4.4 Preparing the Sample Racks and Creating a Scan File Import

Ensure all DNA extracts and reagent blanks (aka DNA sample tubes) are in Tecan compatible tubes and appropriately barcoded.

4.4.1	Place DNA sample tubes in positions 1 through 16 in the sample racks. Use additional sample racks as needed (up 93 sample tubes on 6 racks). Any rack position(s) unfilled by a DNA sample tube must contain an empty tube with a unique "BL" barcode.	

"BL" barcode tubes may be reused; however, each "BL" barcode on the Tecan must be unique.

4.4.2	Use the appropriate script to scan the sample racks and generate a .csv scan file. Import the file into STACS.	

4.5 Master Mix Preparation

4.5.1	Create the master mix based on the volumes below. Equally distribute the master mix between two labeled microcentrifuge tubes. Vortex and quick spin.	

Quantifiler TRIO Master Mix Components

	µL per well*
PCR Reaction Mix	10.0
Primer Mix	8.0
Automation Enhancer	0.018**

*Number of wells = number of samples + 3 controls and appropriate overage (~6)

NOTE: *Master mix must be created for a minimum of 56 wells to prevent pipetting less than 1 ul of automation enhancer.*

**Round the total volume of automation enhancer to 2 decimals as appropriate for the pipette capability.

Vortex and quick spin the prepared Quantifiler® TRIO DNA standard calibrator samples before loading. Calibrator samples may be used for 2 months after thawing for first use.

4.5.2	Place the calibrators and master mix tubes in the Master Mix Rack (see Figure 1): • Positions 1 and 2: Calibrator samples 1:10 (with a "1:10" barcode) and 1:50 (with a "1:50" Barcode), respectively. • Positions 3 and 4: the two tubes containing equal volumes of master mix (with "C1" barcodes). • Positions 5 through 16: empty tubes (with unique "BL" barcodes).	

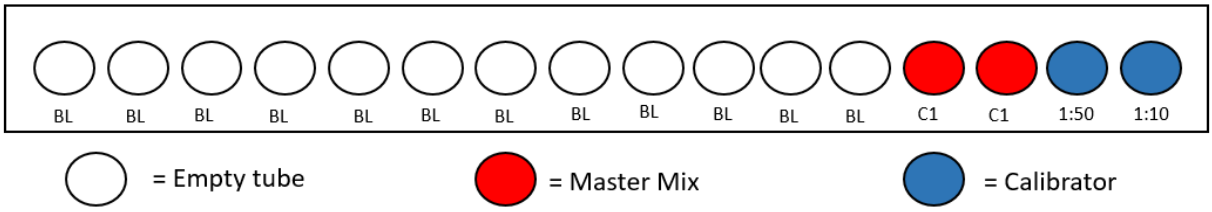

Figure 1 – Positioning for Master Mix Rack

4.6 Quant TRIO Plate Preparation

Ensure all tubes are uncapped prior to run.

4.6.1	Run the current version of the FBI Quantifiler TRIO script, and answer the prompts. The Tecan will add 18 µL of Quantifiler® TRIO master mix and 2 µL of each sample extract or control to the 96 well plate.	

4.6.2	Seal the plate with a clear seal. Quick spin (generally ~2,000 rpm for 5 seconds). Ensure the quant plate barcode is on a side of the plate.	

The seal may be applied with the Thermal Microplate Sealer or, if needed, manually. Ensure that the edges of each well are sealed.

The DNA sample tubes and calibrator sample tubes should be removed from the Tecan deck and capped prior to taking the sealed quant plate to the Amp room.

4.7 Real-Time PCR

4.7.1	Ensure the 7500 and the supporting computer are powered on. Place the sealed plate into the 7500 so that well A1 is in the back-left.	

4.7.2	In the 7500 software, open a new Trio run file. Import the sample setup (.txt file) generated by STACS for the plate ID.	

4.7.3	Save the run file (.eds) with the plate ID in the file name, ensure the 7500 door is closed, and start the run.	

4.8 Data Evaluation

4.8.1	Review the results in the 7500 software. The data will be analyzed using the Virtual Standard Curve (VSC) settings.	

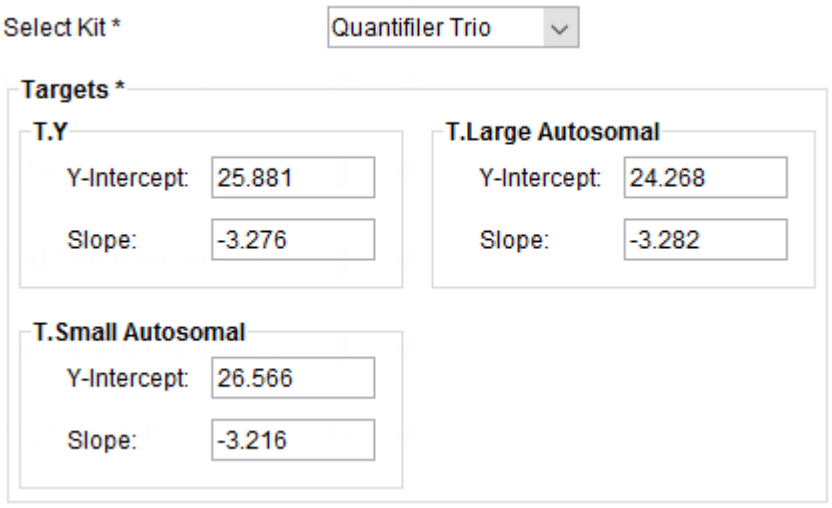

Figure 2 – VSC Settings

4.8.1.1	At least one calibrator sample must meet the C_T parameters for each of the targets below.	

	1:10 (LU Barcode)	1:50 (LO Barcode)
T. Small Autosomal	21.6 - 24.5	23.7 - 26.7
T. Y	21.0 - 25.1	23.1 - 26.9
T. Large Autosomal	19.6 - 22.3	21.8 - 24.0
IPC	26.2 - 30.6	26.1 - 29.6

Calibrator Sample Passing C_T Ranges

If either calibrator sample does not meet the above criteria, both samples should be discarded and new samples thawed for next use.

4.8.2	Export the results to the appropriate folder on the network. (*Select Export from File menu, then choose Results.*) Import the results file (.txt) and run file (.eds) into STACS.	

4.8.3	Check the T. Large Autosomal, T. Small Autosomal and T.Y quantification results and IPC C_T of the Master Mix control. • If the Master Mix control displays a signal, the quantity value detected will be added as a comment in STACS.	

4.8.4	Complete the required fields in STACS. *(If an R^2 value is required, 1 will be entered.)* The plate will be successful if: 1. at least one calibrator sample meets the C_T parameters for each of the targets, and 2. the Master Mix control displays no quantifiable DNA **(or** if any detected value is noted in the comments, it was concluded to be spurious.)	

The Master Mix control should display no quantifiable DNA. If a DNA concentration value appears in the Master Mix control, the concentration values obtained for the RB(s) run on the plate should be examined.

- If one or more of the RBs display no quantifiable DNA, the master mix value can be concluded to be spurious (i.e., not indicative of the presence of adventitious DNA) and the sample data should be used.
- The T. Large Autosomal target is not used for quantification. Values appearing in this target alone should not be considered.

The sample data can be evaluated to determine if any sample should be diluted and/or re-quanted. IPC C_T values are typically between 27 and 30. Undetermined IPC C_T values or values greater than 31 may indicate inhibition.

- Samples that have an indication of possible inhibition may be diluted and re-quanted.
- Samples with excessive DNA (generally >300 ng/µL) should be diluted and re-quanted.

Reagent grade water is used to dilute samples as appropriate. Any dilution(s) made will be recorded in the case notes.

An examiner will review the quant results for each sample. STACS uses the quant results and the default amplification settings to determine the volume of sample to queue for amplification. An examiner should make adjustments to the amplification setup as necessary. Additional guidance is located in the nDNA amplification procedure (i.e., DNA 213).

5 Calculations

The 7500 software uses the standard curve equation:

$$C_T = m\ [\log\ (Qty)] + b$$

where m is the slope and b is the y-intercept as set for the virtual standard curve. The C_T is the cycle threshold measured during the Quantifiler TRIO real-time PCR run and then used by the software to calculate the estimated starting DNA quantity (Qty). While this value is calculated by the software, by rearranging the standard curve equation the Qty can also be calculated with the formula:

$$Qty = 10\text{^}[(C_T\text{-}b)/m]$$

In general, a difference of 1 C_T equates to a two-fold difference in initial template amount. Therefore, if comparing quant data from 2 samples, a sample with 1 C_T higher will have a Qty ~1/2 that of the other sample and conversely a sample with 1 C_T lower will have a Qty ~2x that of the other sample.

6 Sampling

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

Trio may be affected by inhibition when amp kits are not. In such cases, it is possible that samples yielding no result at quant may yield DNA typing results.

9 Safety

9.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. Follow the "Safe Work Practices and Procedures," "Bloodborne Pathogen (BBP) Exposure Control Plan (ECP)," "Personal Protective Equipment Policy," and "Chemical Hygiene Plan" sections of the *FBI Laboratory Safety Manual*.

9.2 Avoid reaching into the Tecan robot while it is running as personal injury could result from moving robot accessories.

10 References

FBI Laboratory Quality Assurance Manual (QAM)

FBI Laboratory Safety Manual

DNA Procedures Manual

Applied Biosystems. *Quantifiler™ HP and Trio DNA Quantification Kits User Guide*, 2017.

Applied Biosystems. *Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Relative Standard Curve and Comparative CT Experiments*. 2010.

Applied Biosystems. *Installation and Maintenance Guide for the Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System*. 2006.

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Green RL, Ines CR, Boland C, and Hennessy LK. 2005. Developmental validation of the Quantifiler real-time PCR kits for the quantification of human nuclear DNA samples. *J. Forensic Sci.* 50:809-825.

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Higuchi R, Fockler C, Dollinger G, and Watson R. Kinetic PCR analysis: Real-time monitoring of DNA amplification reactions. *BioTechnology* 11:1026-1030.

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Rev. #	Issue Date	History
5	02/28/18	1 Adjusted scope 2 Updated software and made necessary adjustments throughout 4.1.1 and 4.1.2 Allowed for both volumes typically used. Added clarification that volume is based on sample or as requested by FE. 4.5.4 Corrected numbering and added allowance for different placement of the barcode based on the Tecan plate holder. 4.8.2.1 Applies to human and male standard curves Appendix A: Edits so that standards in performance verification can be run in duplicate.
6	03/16/20	Complete revision to incorporate changes from Quantifiler DUO to Quantifiler TRIO. Moved RoboScrub to appendix A
7	07/01/21	Editorial revisions throughout. Revised procedure and appendix A for use of virtual standard curve. 4.1: Added vacufuge throughout section 4.3.2: Added steps for water addition on Tecan 5: Added standard curve calculation information

Approval

Redact - Signatures on File

DNA Technical Leader Date: 06/30/2021

DCU Chief Date: 06/30/2021

SBAU Chief Date: 06/30/2021

Appendix A: Quality Control Procedures

1. Instruments

Refer to the DNA procedure for instrument calibration and maintenance (i.e., DNA QA 608) for minimum frequency and additional requirements.

A. General Maintenance of the AB 7500 Real-Time PCR System

Once a year, general maintenance is performed as part of the annual PM. For semi-annual general maintenance, refer to the instructions in the Applied Biosystems *7500/7500 Fast Real-Time PCR System Maintenance Guide* to perform the following:

1. Regions of Interest (ROI) Calibration (Chapter 2)
2. Background Calibration and Optical Calibration (Chapter 3)
3. Dye Calibrations (Chapter 4) for standard dyes VIC and FAM and custom dyes ABY and JUN and, when applicable, for standard dye NED used for the mtDNA qPCR Degradation Assay.

B. Performance Verification of the AB 7500 Real-Time PCR System

The performance verification of the AB 7500 Real-Time PCR System will be accomplished by running both the Quantifiler® TRIO DNA Quantification Kit and, when applicable, the mtDNA qPCR Degradation Assay, as each assay uses different dyes.

1. Refer to the above Quantifiler® TRIO procedures and the procedures for the mtDNA qPCR Degradation Assay (i.e., DNA 404):
    a. Using an in-use lot of Quantifiler® TRIO kit, run a plate containing the two calibrator samples, in triplicate, and appropriate controls.
    b. Using in-use lots of reagents for the mtDNA qPCR Degradation Assay, run a plate containing the mtDNA Quantitative PCR Standard Dilution Series, the HL60 calibrator, and appropriate controls, all in duplicate.

2. The 7500 will be deemed suitable for casework analysis if:
    a. All replicates of the calibrator samples meet the C_T parameters in section 4.8.1.1 and
    b. the slope, Y-intercept, and R^2 values for the mtDNA qPCR Degradation Assay meet the criteria of a passing run:
        i. $R^2 \geq 0.985$
        ii. Slope in the range of -3.200 and -3.600
        iii. Y-intercept in the range of 36.100 and 39.600

3. If the performance verification of the 7500 does not meet the passing criteria for either assay, the unsuccessful plate(s) will be repeated. If the results are still deemed unsuitable, then the Technical Leader will be consulted.

C. General Maintenance of the Tecan Robotic Workstation

RoboScrub cleaning should be performed weekly, generally at the end of a workday:

1. Make ~3.5 L of diluted Liquinox (see instructions on the label of the bottle for preparation)
2. ~3.5 L purified water in a separate container is needed
3. Run the RoboScrub Clean script, and follow the prompts

D. Performance Verification of the Tecan Robotic Workstation

1. An Artel MVS Multichannel Verification System and NIST traceable standards will be used to test the accuracy and precision of the liquid handling by the Tecan. Refer to the *Artel MVS Multichannel Verification System User Guide* for operation of the Artel MVS.

2. The Tecan Robotic workstations are typically configured with eight (8) fixed tips and there are multiple volumes aliquoted during each procedure. A minimum of 6 repetitions will be performed with each tip for each volume.

3. The results must be within the tolerance limits set by DCU for each volume. At times, it may be necessary to modify/optimize the Tecan liquid class parameters (e.g., offset and factor).

4. If the performance verification of the Tecan does not meet the above listed criteria, the performance verification will be repeated. If the results are still deemed unsuitable, then the Technical Leader will be consulted.

2. Critical Reagents

Refer to the DNA procedure for reagent purchasing, preparation and records (i.e., DNA QA 609) for additional requirements.

A. Preparation of Applied Biosystems Quantifiler® TRIO DNA standard Calibrator Samples

Calibator samples will be prepared from the Quantifiler® TRIO Standard Stock (~100 ng/µl). Calibrator samples will be stored frozen, until thawed, then may be stored refrigerated and used for up to two months.

1. Prepare a 1:10 dilution (~10 ng/µl) by adding 30 µl of the Quantifiler® TRIO Standard Stock and 270 µl of TE^{-4}.

2. Prepare a 1:50 dilution (~2 ng/µl) by adding 6 µl of the Quantifiler® TRIO Standard Stock and 294 µl of TE^{-4}.

B. Qualification of Applied Biosystems Quantifiler® TRIO DNA Quantification Kit and Calibrator Samples

1. Each new lot of Quantifiler® TRIO kits will be evaluated by running a standard dilution series (50, 5, 0.5, 0.05, 0.005 ng/µl) of the NIST SRM 2372 Component A (in duplicate), the prepared calibrator samples, and appropriate controls (include a TE^{-4} control to account for prepared calibrator sample diluent) and analyzed with the VSC.

2. The new Quantifiler® TRIO kit lot will be deemed suitable for casework analysis if:
   a. both replicates of three of the five SRM dilutions fall within 30 percent of the expected values for each of the three targets (small autosomal, large autosomal, and Y) and
   b. both calibrator samples meet the C_T parameters in section 4.8.1.1.

3. If the SRM dilutions do not meet the passing criteria, new dilutions will be prepared and the plate will be re-run. If the calibrator samples do not meet the passing criteria, the dilutions will be adjusted and the plate will be re-run. If the results are still deemed unsuitable, the Technical Leader will be consulted. The VSC will be reevaluated if there is an indication that it is no longer working appropriately.